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Biological Features and Chemical Components of Red Pigments Produced by A Novel Ascomycete Fungus, *Gelatinomyces siamensis* in Liquid Media

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Red pigments of a newly discovered fungus, *Gelatinomyces siamensis* recently characterized and classified in Leotiomycetes, *Incertae Sedis* were produced in liquid media. The role of this apparent reddish color was questioning. Therefore, the red pigments produced by the fungus were extracted with ethyl acetate and butanol to obtain two fractions of crude substances to seek for biological activities. The results suggested crude extract derived from ethyl acetate was significantly greater in antioxidation and total phenolic compounds compared with the extract due to butanol. Additionally, both crudes were screened for other biological assays. While the butanol fraction was inactive in all tests, the ethyl acetate fraction was active against phytopathogenic fungus, *Magnaporthe grisea* with minimal inhibitory concentration (MIC) of 50.00 μg/ml and against cancer cells (NCI-H187-Small cell lung cancer) with half maximal inhibitory concentration (IC_{50}) of 20.44 μg/ml. This could provide supports to further investigate the pigment derived from ethyl acetate extraction. Moreover, preliminary identification of chemical components were performed using gas chromatography mass spectrometry (GCMS) implying the compounds contained in both fractions were mainly acids and components associated with pigment producing pathways.

Key words: Antioxidation, bioactivity, GCMS, gelatinous ascomycetes, Phenolic, Pigmented fungi.

A broad range in colors of fungal pigments such as yellow, orange, pink and brown due to various molecules such as quinones (anthraquinones and naphthaquinones), dihydroxy naphthalene melanin (a complex form of polyketides) and flavin compounds (riboflavin) have been reported and well characterized[1]. A number of studies investigating colorants secreted by fungi have indicated that they are particularly in ascomycota and they are also alternative sources of safe and certified ingredients for food, antibiotics, alcoholic products, enzymes, organic acids, pharmaceuticals and dietary supplements[2-4]. Apart from these, natural colorants from fungi are broadly utilized especially as dyes in wooden products and textiles[5].

Colorants derived from fungi have been historical in their applications, for example, to naturally stain woods with dyes produced by different fungi like *Scytalidium cuboideum, Monascus purpureus, Trametes versicolor, Xylaria polymorpha, Inonotus hispidus, Arthrographis cuboidea* and *Acer negundo* [5, 6]. Moreover, some ascomycete fungi like *Penicillium* and *Epicoccum* have been largely studied regarding to their secreted pigments giving wide color spectrum ranging in yellow, orange and red[7]. Due to the various color shades of pigments these fungi

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produce, they are expected to be future natural colorants[8]. Current products available in markets are mainly from synthetic chemicals however it is predicted that the growth of natural-derived colorants are increasing at the rate of 5-10% annually and color-producing fungi are the candidates aside from plants and algae as the color producers to substitute the synthetic ones[9].

Gelatinomyces siamensis Sanoamuang, Jitjak, Rodtong & Whalley, a fungus inhabits on bamboo branch collected from Nam Nao National Park, Thailand, recently classified as a novel ascomycete fungus in Leotiomycetes *Incertae Sedis*, has also been found to produce two types of red pigments into media, water-soluble and water-insoluble[10]. For the soluble pigment, the fungus secretes it as droplets which change the color of the liquid media from light yellow into pink, then red and finally dark red. Red crystals as water-insoluble pigment are also seen. The fungal pigments in some cases termed as secondary metabolites are generated for certain reasons, most likely having biological activities. For instance, a group of pigments, naphthoquinones serves as mycotoxins and red pigments containing anthraquinone-like structures from *Isaria farinosa* (Holmsk.) perform antibiotic activities and toxicity[11]. Similarly, *G. siamensis* was expected its secreted red pigments were for at least one of these reasons.

Apart from identification and classification, it was sensible for this ascomycete fungus, to examine the obvious pigments. This could also decipher a question why the pigments always exist once the fungus has established in the media. The aim of this study was therefore to seek for some biological activities and constituents of crude extracts of the red pigment to provide further supports for extensive studies associated with the pigments.

MATERIALS AND METHODS

Production and extraction of crude extract of red pigment

The one-month -old fungal isolates, KKUK1 and KKUK2, from potato dextrose agar (PDA) slant were aseptically transferred into 2-L potato dextrose broth (PDB) contained in culture bottles and they were incubated for 30 days at 25 °C to let them produce red pigments into the broth. After that, the mycelia were filtered and the broth was collected. The selected extraction solvents were ethyl acetate and butanol to obtain compounds in the pigmented broth. Then, they were evaporated to expel the solvents at 45 °C using rotary evaporator and the crude extracts from these two solvents were separately kept at 25 °C for further assays.

Measurement of 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH)-radical scavenging activity

DPPH radical scavenging activity was assessed[12]. In this assay with three replicates, 50 µL of crude extracts with different concentrations derived from ethyl acetate (1 – 7 µg/ml) and butanol (25 – 175 µg/ml) was added with 1.0 mL of 0.4 mM methanolic-DPPH and followed with methanol to 5.0 mL. The mixture was vigorously shaken using vortex and left for 30 min at room temperature in a dark condition. The scavenging effect on the DPPH radical was read using UV/VIS spectrophotometer (UV-1700 Pharamspec) at 517 nm. The radical scavenging activity was expressed as the radical scavenging percentage using the following equation, Percentage (%) of DPPH radical scavenging = (1-(Ac-As)) × 100 where Ac is absorbance of control and As is absorbance of sample solution. The DPPH solution without sample solution was used as control. Half maximal effective concentration (EC_{50}) value is the concentration of sample required to scavenge 50% of DPPH free radical and it was calculated from the plotted graph of radical scavenging activity against the concentrations of extracts. Ascorbic acid and tocopherol were used as positive controls. To compare the means of EC_{50} values from each sample, *t*-test was performed with 95% confidence.

Ferric reducing ability of plasma assay (FRAP)

Ferric reducing ability of plasma assay (FRAP) was another method to evaluate the antioxidant potential[13]. The method consisted of FRAP reagents which were 1) acetate buffer 300 mM pH 3.6, dissolving 3.1 g sodium acetate trihydrate in 16 ml of glacial acetic acid and then adjusting the volume to 1 L with distilled water, 2) TPTZ (2, 4, 6-tripyridyl-s- triazine), 10 mM in 40 mM HCl and $FeCl_3.6H_2O$, 20 mM. The working FRAP solution was made by combining these 3 solutions in the ratio of 10: 1: 1 respectively just

prior to the test. Standard reagent was $FeSO_4 7H_2O$, 0.1 - 1.5 mM in methanol. Briefly, 3.6-ml FRAP solution was added to distilled water (0.4 mL) and then mixed with fungal extracts (3 µg and 30 µg from ethyl acetate and butanol respectively) and incubated at 37ÚC for 10 min. The absorbance of the reaction mixture was measured at 600 nm. For construction of the calibration curve, five concentrations of $FeSO_4 7H_2O$ (0.10, 0.40, 0.80, 1.00, 1.12mM) were used and the absorbance values were measured as previous experiment, DPPH assay[13]. This was performed in three replicates. The statistical analysis to compare the results was *t*-test with 95% confidence.

Determination of total phenolic content (TPC)

Total Phenolic Content (TPC) in fungal extracts was determined by using Folin-Ciocalteu colorimetric methods based on the redox reaction[14]. Determinations were carried out in triplicate averaged and calculated from a standard curve of tannic acid. An aliquot of 20 µL of each sample solution and blank was taken into separated cuvettes and 1.58 ml of water with 100 µl of 2N Folin-Ciocalteu solution was added and homogenized. Additionally, 300 µL of 20% (w/v) Na_2CO_3 was added and well mixed by agitating. The sample was incubated for 2 h at room temperature. The absorbance (Abs) was measured at 510 nm in a UV/VIS spectrophotometer (UV-1700 Pharamspec) and was plotted against concentration of the fungal extracts with methanol as blank. TPC in fungal extracts was calculated and termed as tannic acid equivalents (TAE) using the following equation: C = cV/m where C is the total content of phenolic compounds, mg TAE per g dry extract (dE), c as the concentration of tannic acid obtained from the calibration curve, mg/ml, V as the volume of extract, ml and m is the weight of extract[15]. The *t*-test analysis to compare the resulted values was conducted with 95% confidence.

Biological tests

To further seek for other biological activities, the crude extracts derived from ethyl acetate and butanol were tested for a set of biological activities against pathogenic microorganisms and cancer cell lines, 1) anti-*mycobacterium tuberculosis* (Anti -TB) H37Ra strain employing the method of green fluorescent protein microplate assay (GFPMA) using 0.5% DMSO as negative control and rifampicin, streptomycin, isoniazid, ofloxacin and ethambutol as positive controls, 2) anti-phytopathogenic fungi against *Magnaporthe grisea* (T.T. Hebert) M.E. Barr. using a fluorometric method, 5(6)-Carboxy fluorescene diacetate (CFDA) assay with 5.0% DMSO as negative control and amphotericin B as positive control, 3) anti-malaria (*Plasmodium falciparum* William H. Welch, K1 Strain) via microculture radioisotope technique (0.1% DMSO as negative control and dihydroartemisinine and mefloquine as positive control), 4) neuraminidase (NA) inhibition assay employing fluorometric determination (MUNANA-based enzyme inhibition assay) with 1% DMSO for determining influenza susceptibility to the NA inhibitor (NAI) antivirals and oseltamivir carboxylate as negative and positive control respectively, 5) anti-cancer (NCI-H187-Small cell lung cancer) using resazurin microplate assay (REMA) technique with 0.5% DMSO as negative control and two positive ones, ellipticine and doxorubicin, 6) anti-bacterial against *Bacillus cereus* Frankland & Frankland (Anti-*B. cereus*) and anti-cancer (MCF7-breast cancer) through similar method and negative control as the anti-small lung cancer activity with vancomycin as positive control for anti- *Bacillus* test and two positive controls for anti-breast cancer assay, tamoxifen and doxorubicin. The final concentration of the crude extracts used for all tests were 50 µg/ml which was the maximum concentration considered as being active except for anti-malarial and neuraminidase (NA) inhibition, which were at 10 and 100 µg/ml respectively. The crude extracts of the fungi were sent to National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand, to test these biological activities.

Gas chromatography mass spectrometry (GCMS)

Disc and sample preparation

The 6-mm diameter discs were prepared from filter paper, Whatmann No. 1 and the paper discs were sterilized by autoclave at 121 °C. After the sterilization discs were dried at 60 °C. Then solvent extract discs and control discs were prepared. Crude extracts, 1 mg from both ethyl acetate and butanol were dissolved in 1-mL methanol, HPLC grade[16].

GCMS Analysis

The GCMS analysis of the *G. siamensis*

crude extracts was performed using a Clarus 500 Perkin Elmer gas chromatography with a Elite-5 capillary column (5% Diphenyl 95% dimethyl poly siloxane) (30nm~0.25mmID~0.25µmdf) and mass detector was operated in EI mode. Helium was the medium gas at a flow rate of 1 ml/min. The temperature for injector was set at 200 °C and the oven temperature was programmed as follows, 60 °C 15 min, then gradually increased to 280 °C at 3 min[16]. The identification of components was based on comparison of their mass spectra with Wiley, PMW_TOX2 and NIST147 libraries.

RESULTS

Antioxidant activities and total phenolic compounds

The fungal extract derived from ethyl acetate exhibited great potential to inhibit free radicals (EC_{50} = 9.90 ± 0.022 µg/ml) which meant that the crude extract from ethyl acetate could be able to inhibit 50% of free radicals from DPPH at concentration 9.90 µg.mL^{-1}. On the other hand, EC_{50} of fungal extract derived from butanol had significantly weak ability of free radical inhibition, 204.53 ± 3.083 µg/ml compared with those of ascorbic acid, tocopherol and ethyl acetate extract as shown in Table 1.

Table 1. Effective concentration to inhibit 50% of free radicals (EC_{50}) of 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) -radical scavenging activities of fungal crude extracts. Ascobic acid and tocopherol are positive controls

Substance	EC_{50} (µg/ml)
Ascorbic acid	3.67 ± 0.051[a]
Tocopherol	7.57 ± 0.014[b]
Crude extract derived from ethyl acetate	9.90 ± 0.022[c]
Crude extract derived from butanol	204.53 ± 3.083[d]

Similar superscript letters on the values indicate no significant difference (P ≤ 0.05) by t- test.

Likewise, according to Table 2, another method to measure the power of antioxidant activity called FRAP gave similar results as DPPH method. It was clearly seen that the fungal extract from ethyl acetate contained significantly higher ability of anti oxidation, 261.084 ± 3.413 µg/ml, calculated from equation, y = 0.203x + 0.011 derived from the standard curve (not shown) compared with that of the extract obtained from butanol, 38.624 1.232 µg/ml.

Table 2. Ferric reducing ability of plasma (FRAP) assay activity of fungal crude extract

Substance	Concentration (µg/mg)
Crude extract derived from ethyl acetate	261.08 ± 3.413[a]
Crude extract derived from butanol	38.62 ± 1.232[b]

Similar superscript letters on the values indicate no significant difference (P ≤ 0.05) by t- test.

Total phenolic substances were expected to be one of significant compounds associated with antioxidant activity in the crude extracts. Hence, the measurement of total phenolic compounds was performed. The result was also corresponding to the anti oxidation abilities i.e. due to values calculated from the equation, y = 32.93x + 0.020 from the standard curve (not shown), the crude extract from ethyl acetate contained total phenolic compounds (624.320 ± 65.254 mg/g TAE) which was significantly greater than that from butanol which was only at 22.883 ± 1.022 mg/g TAE as shown in Table 3.

Table 3. Total phenolic compounds in fungal crude extracts equivalent to tannic acid

Substance	Concentration (µg/mg)
Crude extract derived from ethyl acetate	624.32 ± 65.254[a]
Crude extract derived from butanol	22.88 ± 1.022[b]

Similar superscript letters on the values indicate no significant difference ($P \leq 0.05$) by t- test.

GCMS analysis

The total compounds detected by gas chromatography were shown in Table 4 and 5. The compositions of crude extract from ethyl acetate detected by GCMS compared to the libraries suggested that the major predicted compounds were stearic acid and n-hexadecanoic acid made up of 61.79% and 28.63% respectively due to peak area. Moreover, there were a number of minor substances possessing phenolic properties including other acids, acid derivatives and cyclic compounds as listed in Table 4. Meanwhile, the GCMS profiling of the compounds in crude extract obtained from butanol showed that hexyl butanoate, 2-(2-hydroxyethoxy) ethyl ester octadecanoic acid, alpha-hydroxysuccinic acid and

Table 4. List of compounds due to their mass spectra contained in crude extract derived from ethyl acetate compared to the libraries. Retention time, molecular formula and weights and peak areas are also exhibited

Peak no.	Retention Time	Name of compound	Molecular formula	Molecular weight	Proportion (%)
1	4.232	1-Hydroxylamino-2-nitro-1-phenylethane	$C_8H_{10}N_2O_3$	182	0.05
2	4.990	4-Acetamido-N-methyl-3-nitrobenzamide	$C_{10}H_{11}N_3O_4$	237	0.04
3	6.825	1,1-Dimethyltetramethoxydisiloxane	$C_6H_{18}O_5S_{12}$	226	0.04
4	8.725	3-Methoxy-4-Trimethylsilyloxy-Benzaldehyde-O-Methyloxime	$C_{12}H_{19}O_3SI$	253	0.05
5	10.255	Tridecane	$C_{13}H_{28}$	184	0.03
6	11.264	3-Acetoxy-3-hydroxypropionic acid	$C_6H_{10}O_5$	162	0.09
7	14.134	Hydroxyethane-1,2-dicarboxylic acid	$C_4H_6O_5$	134	0.42
8	18.293	1,3-Benzenediol	$C_7H_8O_2$	124	0.22
9	20.759	Benzeneacetic acid	$C_9H_{10}O_3$	166	0.20
10	21.109	2,4-bis,1,1-dimethylethylphenol	$C_{14}H_{22}O$	206	0.05
11	22.122	Myristic acid	$C_{14}H_{28}O_2$	228	0.18
12	24.126	Cyclooctasiloxane	$C_{16}H_{48}O_8Si_8$	592	0.03
13	25.340	Methyl 9-methylundecanoate	$C_{13}H_{26}O_2$	214	0.03
14	26.045	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	0.58
15	27.869	Palmitic acid	$C_{16}H_{32}O_2$	256	0.12
16	28.127	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl)ester	$C_{16}H_{22}O_4$	278	0.08
17	28.999	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.02
18	29.730	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	28.63
19	30.687	Pentadecanoic acid	$C_{16}H_{32}O_2$	256	0.05
20	30.834	Palmitic acid	$C_{16}H_{32}O_2$	256	0.06
21	31.291	Pamitinic acid	$C_{16}H_{36}O_2$	284	0.49
22	32.321	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	0.35
23	32.498	9,12- Octadecanoic acid	$C_{18}H_{32}O_2$	280	2.30
24	32.563	9- Octadecanoic acid	$C_{18}H_{34}O_2$	282	3.33
25	33.030	Stearic acid	$C_{18}H_{36}O_2$	284	61.79
26	35.964	Eicosanoic acid	$C_{20}H_{40}O_2$	312	0.60

succinic acid were the major predicted compounds composed of 16.33%, 15.01%, 10.55% and 10.47% due to peak area respectively. Similar to ethyl acetate extract, there were also other compounds performing phenolic properties, acids, cyclic molecules and acid derivatives as shown in Table 5.

Table 5. List of compounds due to their mass spectra contained in crude extract derived from butanol compared to the libraries. Retention time, molecular formula and weights and peak areas are also exhibited

Peak No.	Retention time	Name of compound	Molecular formula	Molecular weight	Proportion (%)
1	10.255	2,4-Dimethyldecane	$C_{12}H_{26}$	170	0.23
2	10.544	1-t-butyldimethylsilylthio-2-methylpropane	$C_{10}H_{24}SSI$	204	0.18
3	10.651	Butanedioic acid	$C_5H_8O_4$	132	1.60
4	11.221	3-Acetoxy-3-hydroxypropionic acid	$C_6H_{10}O_5$	162	1.60
5	12.849	2-Methyl-2,3-epoxy-1-butanol	$C_5H_{10}O$	86	1.47
6	14.131	Malic acid	$C_6H_{10}O_5$	134	0.21
7	14.373	Hexyl butanoate	$C_{10}H_{20}O_2$	172	2.31
8	15.391	Succinic acid	$C_4H_6O_4$	118	10.47
9	15.999	1-ethyl 4-methyl succinate	$C_7H_{12}O_4$	160	0.60
10	17.587	Ethyl hydrogen succinate	$C_6H_{10}O_4$	146	5.04
11	18.050	Dimetyl ester	$C_6H_{10}O_5$	162	1.01
12	18.354	Hexyl butanoate	$C_{10}H_{20}O_2$	172	16.33
13	19.999	Alpha-Hydroxysuccinic acid	$C_4H_6O_5$	134	10.55
14	20.175	Methyl 1-ethyl-2(E)-butenyl ether	$C_7H_{14}O$	114	1.10
15	21.114	2,4-di-tert-butylphenol	$C_{14}H_{22}O$	206	0.32
16	22.044	Di-n-butyl succinate	$C_{12}H_{22}O_4$	230	0.22
17	22.126	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	0.12
18	22.253	4-Hydroxyphenylacetic acid	$C_8H_8O_3$	152	0.35
19	22.818	Hexadecane	$C_{16}H_{34}$	226	0.62
20	23.744	1-Propyl-2-azepanol 1-oxide	$C_9H_{19}NO_2$	173	1.78
21	23.818	2-Isocyanato-2-methyl-propane	C_5H_9NO	99	1.77
22	24.131	Hexadecamethyl-cyclooctasiloxane	$C_{16}H_{48}O_8SI_8$	592	0.17
23	25.162	Octylphenlisomer	$C_{14}H_{22}O$	206	0.09
24	25.325	n-Nonylphenol	$C_{15}H_{24}O$	220	0.22
25	25.800	1-(Hexyloxy)heptane	$C_{13}H_{28}O$	200	0.08
26	25.876	4-(n-Butoxyphenyl)acetic acid	$C_{12}H_{16}O_3$	208	0.25
27	26.046	Myristic acid	$C_{14}H_{28}O_2$	228	0.35
28	26.706	Octadecane	$C_{18}H_{38}$	254	0.95
29	27.140	1,1,1,5,7,7,7-Heptamethyl-3,3-bis (trimethylsiloxy)tetrasiloxane	$C_{13}H_{40}O_5SI_6$	444	0.22
30	29.619	Palmitic acid	$C_{16}H_{32}O_2$	256	4.87
31	29.777	Di-n-butyl phthalate	$C_{16}H_{22}O_4$	278	0.64
32	30.216	Eicosane	$C_{20}H_{42}$	282	1.02
33	30.507	1-Hexadecanol	$C_{16}H_{34}O$	242	0.13
34	32.326	Methyl ester octadecanoic acid	$C_{19}H_{38}O_2$	298	0.20
35	32.476	14-Methyl-8-hexadecyn-1-ol	$C_{17}H_{32}O$	252	0.36
36	32.541	Heptadecene-8-carbonic acid	$C_{18}H_{34}O_2$	282	0.55
37	32.469	5-Methylhenicosane	$C_{22}H_{46}$	310	0.44
38	32.897	2-(2-hydroxyethoxy)ethyl ester octadecanoic acid	$C_{22}H_{44}O_4$	372	15.01
39	33.412	Heneicosane	$C_{21}H_{44}$	296	0.88
40	36.476	Octacosane	$C_{28}H_{58}$	394	0.39
41	36.597	Cyclodocosanolie	$C_{22}H_{42}O_2$	338	0.25

Biological tests of crude extracts

Seven biological assays were selected. Firstly, anti-*M. tuberculosis* (Anti -TB) H37Ra strain using green fluorescent protein, *minimal inhibitory concentration (*MIC) values of positive controls were 0.003 µg/ml (rifampicin), 1.25 µg/ml (streptomycin), 0.047 µg/ml (isoniazid), 0.781 µg/ml (ofloxacin) and 1.88 µg/ml (ethambutol). Secondly, anti-phytopathogenic fungal activity against *M. grisea* through 5(6)-carboxy fluorescene diacetate (CFDA) assay, MIC value of positive control was 1.56 µg/ml (amphotericin B). Anti-malaria (*P. falciparum*, K1 Strain) with microculture radioisotope technique, IC_{50} values of positive controls for this assay were 2.51 nM and 0.0262 µM for dihydroartemisinine and mefloquine respectively. Neuraminidase (NA) inhibition assay employing fluorometric determination (MUNANA-based enzyme inhibition assay), the IC_{50} value of positive control was 0.741 nM (oseltamivir carboxylate). Anti-cancer (NCI-H187-small cell lung cancer) using resazurin microplate assay (REMA), 0.726 µg/ml and 0.108 µg/ml were IC_{50} values of the positive controls, ellipticine and doxorubicin respectively. The following assay was anti-bacterial activity against *B. cereus* (Anti-*B. cereus*) via resazurin microplate assay (REMA) with vancomycin as positive control performing MIC value of 2.0 µg/ml. Lastly, anti-cancer (MCF7-breast cancer) due to resazurin microplate assay (REMA), the IC_{50} numbers of positive controls were 5.86 µg/ml and 8.87 µg/ml for tamoxifen and doxorubicin respectively.

The crude extracted with ethyl acetate not only showed high anti-oxidation and total phonolic compounds but also exhibited being active to two of seven anti biological activities which were anti-phytopathogenic activity against *M. grisea* (MIC = 50.00 µg/ml) and anti-cancer (NCI-H187-Small cell lung cancer) with 20.44 µg/ml as its IC_{50} value. However, the remaining assays were inactive as shown in Table 6. With positive results in the two tests, anti *M. grisea* and anti-Small cell lung cancer, it is optimistic that certain compounds contained in this fraction extracted with ethyl acetate could be able to act against the pathogenic fungal and the cancerous lung cells. However, the extract from butanol did not show any positive result implying that the compounds in this fraction did not have strong ability to pronounce significant bioactivities.

Table 6 The biological tests of the *Gelatinomyces siamensis* crude extract derived from Ethyl acetate which is active to act against *Magnaporthe grisea* and NCI-H187-Small cell lung cancer

Test	Result
Anti-Mycobacterium tuberculosis (Anti -TB) H37Ra strain	Inactive
Anti-phytopathogenic fungal activity against *Magnaporthe grisea*	Active (MIC = 50.00 µg/ml)
Anti-malaria (*Plasmodium falciparum*, K1 Strain)	Inactive
Anti-Cancer (MCF7-breast cancer)	Inactive
Anti-Cancer (NCI-H187-Small cell lung cancer)	Active (IC_{50} = 20.44 µg/ml)
Anti-bacterial against *Bacillus cereus* (Anti-*B. cereus*)	Inactive
Neuraminidase (NA) inhibition assay	Inactive

DISCUSSION

The total phenolic compounds in crude extracts derived from ethyl acetate and butanol was related to the level of antioxidant activities i.e. the phenolic content was higher in the ethyl acetate fraction which was also greater in the level of anti-oxidant power compared with the butanol-derived fraction. This suggested that compounds with low-to-medium polarity from the pigments drawn by ethyl acetate contained highly active molecules. Conversely, those dissolved in butanol were high-polar substances but their performances according to the bioassays were not very promising. This was corresponding to a study previously reported[17]. The researchers used three different solvents to extract crude substances from culture medium of *Antrodia cinnamomea* Chang & Chou. They found the crude extract derived from ethyl acetate showed the highest antioxidant activity due to DPPH method[17]. So that, ethyl acetate was

the effective organic solvent that could be able to draw the potential components significant to bioactivities from the pigment[18]. Besides, FRAP method also yielded the same outcome as DPPH assay i.e. both antioxidative tests indicated the extract from ethyl acetate were significantly greater in free radical disposal. Therefore, the crude extract obtained from this solvent could contain bioactive compounds which are required further investigation using higher disciplines in analytical chemistry.

The results of selected bioassays suggested certain potentials hidden in crude extract particular in ethyl acetate fraction by reason of its ability to be against the lung cancer cells and the rice blast fungus. These bioactivities are also taken into account for further chemical separation until the pure compounds in the crude extract are derived. Despite negative activity against the tests of the crude extract derived from butanol, opportunity to have some active components in this fraction is possible as the crude consists of numerous constituents, both active and inactive compounds but the proportion of inactive compounds outnumbering the active ones could diminish the performances[19]. It is thus interesting to employ protocols in analytical chemistry for future study on separation and determination of chemical components in the pigmented extracts particularly in the crude derived from ethyl acetate. Prior to in-depth chemical investigation, the preliminary investigation to predict groups and a brief number of chemical components in the crude fractions was also conducted.

As the results of antioxidant activities, phenolic content and biological assays, crude extract derived from ethyl acetate became interesting for further study which mainly consisted of stearic acid and hexadecanoic acid according to the comparison with mass spectra in libraries. Various fatty acids including polyunsaturated stearic acid were detected via GCMS which are substantial for membrane flexibility[20, 21]. Not only being a constituent of plasma membrane structure, these fatty acids are also a group of metabolites produced by fungi ecologically as endophytes[22]. Likewise, n-hexadecanoic or palmitic acid is one of the components crucial for plasma membrane formation and also an anti-inflammatory agent to inhibit phospholipase A(2) which is an enzyme initiating the inflammation. This is the indication that one of the major compounds identified by GCMS has pharmaceutical potential[23].

The GCMS analysis used in this study is limited to compounds that are able to volatile[24]. However, it can still give some clues of the metabolic pathways associate with pigment synthesis. A large number of acids related to cellular biosynthesis were detected in both fractions. There is a report suggesting that acids found in fungi can be originated from different pathways e.g. pulvinic acids, arylpyruvic acids, butenolides, 4-hydroxybenzoic acid, acetate–malonate and carboxylic acid pathway. In this study, the compounds identified in the extracts were corresponding to these acid groups and pathways[25]. Moreover, another red pigment producing fungus has also been reported regarding to metabolites responsible for fungal pigment synthesis e.g. the evidence shows the *Penicillium* sp. isolate has the ability to synthesize glutamate based polyketide pigment which is also seen red in broth[26]. Apart from that, *Monascus ruber* Tiegh. has been found to have tetraketide as the first compound for citrinin, claimed as an anti-bacteria mycotoxin and to have red pigments production during its development. Briefly related to red pigment production, beginning with tetraketide, the pathway separates into two different pathways which are tetraketide to penaketide to hexaketide to red pigment, and tetraketide to citrinin. Various enzymes involved in the reaction at the tetraketide level would account for a different production of pigments from a hexaketide[27, 28]. The red pigments produced by *G. siamensis* are also expected to have similar synthetic pathways as well. This leads to additional investigation of the crude extract and also synthetic pathways associated with its pigments. This report on the compounds and bioactivities could indicate the effectiveness of pigments which is promising to purify especially the pigment from ethyl acetate extraction for further applications.

CONCLUSION

The study demonstrated the optimistic biological activities of the red pigment produced by the novel fungus, *G. siamensis* derived from

ethyl acetate extraction as being a great antioxidative agent, containing a great proportion of phenolic content and being active against rice blast fungus and lung cancer cells. As the results of these, it is then interesting to further examine the pigment derived from ethyl acetate for future beneficial applications and could decipher the biological role including the reason why the production of the red pigments is stimulated when the fungus is cultured *in vitro* or even inside the fruiting bodies found in nature.

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